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INDUSTRIAL BIOTECHNOLOGY DEPARTMENT

Ref : LCH/tp/2003-004

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Object : patent application US09/673,288

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**To whom it may concern,**

I, the undersigned Laurent Chevalet,

domiciled : 1241 route de Ferrières – (F) 74350 CUVAT,  
presently working for the Pierre Fabre Group as Head of the Industrial Biotechnology  
Department of the Centre d'Immunologie based in Saint-Julien en Genevois, France,  
having the following qualification : Engineer in Biotechnology, PhD in Microbial Genetics,

hereby state and certify that :

- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides Trp5 and Trp2 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 1, having a sequence encompassing positions 1 to 503 of the sequence described in appendice 1.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides Trp3 and Trp4 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 2, having a sequence encompassing positions 498 to 1335 of the sequence described in appendice 1.
- any person skilled in the art ligating the above mentioned fragments 1 and 2 will generate a DNA fragment having the sequence described in appendice 1.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR1 and TrpR2 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 3, having a sequence encompassing positions 1 to 520 of the sequence described in appendice 2.
- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR3 and TrpR4 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 4, having a sequence encompassing positions 508 to 843 of the sequence described in appendice 2.

- any person skilled in the art performing an amplification reaction, using (i) the oligonucleotides TrpR5 and TrpR6 and (ii) E. coli chromosomal DNA as template, will generate a DNA fragment, hereby named fragment 5, having a sequence encompassing positions 838 to 1468 of the sequence described in appendice 2.
- any person skilled in the art linking fragments 3 and 4 together and ligating them with fragment 5 will generate a DNA fragment having the sequence described in appendice 2.
- the two following publications : Deeley & Yanofsky (1981), and Gunsalus & Yanofsky (1980) cited in the above-mentioned US patent application provide evidence of the sequences described in appendices 1 and 2.

Made in Saint-Julien en Genevois, February 27<sup>th</sup>, 2003.

A handwritten signature in black ink, appearing to read "Deeley", with a stylized flourish extending from the end.

**Appendice 1 : sequence used to inactivate the tryptophanase tnaA gene by insertion of a stop codon (construction of ICONE 100)**

Nucleotides 1 to 275 : sequence of the E. coli tnaA promoter

Nucleotides 276 to 1335 : 5' part of the E. coli tnaA gene coding sequence

Nucleotides 276 to 278 : start codon of the E. coli tnaA gene

Nucleotides 495 to 503 : stop codon inserted in the E. coli tnaA gene followed by an XbaI restriction site

GTGTGACCTCAAAATGGTTCAATATTGACAACAAAATTGTCGATCACCGCCCTTGATTGCCCCTTCTGTAGCCAT  
CACCAGAGCCAAACCGATTGATTCAATGTGTTCTATTTGTTTGCTATATCTTAATTTTGCCTTTTGCAAAGGTCA  
TCTCTCGTTTTATTTACTTGTTTTAGTAAATGATGGTGCTTGCATATATATCTGGCGAATTAATCGGTATAGCAGA  
TGTAATATTCACAGGGATCACTGTAATTAATAAATAAATGAAGGATTATGTAATGGAAGAACTTTAAACATCTCCCTG  
AACCGTTCCGCATTCGTGTTATTGAGCCAGTAAACGTACCACTCGCGCTTATCGTGAAGAGGCAATTATTAAAT  
CCGGTATGAACCCGTTCTCTGCTGGATAGCGAAGATGTTTTTATCGATTTACTGACCGACAGCGGCACCGGGGCGG  
TGACGCAGAGCATGCAGGCTGCGATGATGCGCGGCGACGAAGCCTAATCTAGACAGCGGCAGTCGTAGCTACTAT  
GCGTTAGCCGAGTCAGTGAAAAATATCTTCGGTTATCAATACACCATTCCGACTCACCAGGGCCGTGGCGCAGAG  
CAAATCTATATTCCGGTACTGATTAAAAACGCGAGCAGGAAAAAGGCCTGGATCGCAGCAAAATGGTGGCGTTC  
TCTAACTATTTCTTTGATACCACGCAGGGCCATAGCCAGATCAACGGCTGTACCGTGCGTAACGTCTATATCAAA  
GAAGCCTTCGATACGGGCGTGCGTTACGACTTTAAAGGCAACTTTGACCTTGAGGGATTAGAACGCGGTATTGAA  
GAAGTTGGTCCGAATAACGTGCCGTATATCGTTGCAACCATCACCAGTAACCTCTGCAGGTGGTCAGCCGGTTTCA  
CTGGCAAACCTAAAAGCGATGTACAGCATCGCGAAGAAATACGATATTCCGGTGGTAATGGACTCCGCGCGCTTT  
GCTGAAAACGCCTATTTTATTAAGCAGCGTGAAGCAGAATACAAAGACTGGACCATCGAGCAGATCACCCGCGAA  
ACCTACAAATATGCCGATATGCTGGCGATGTCCGCCAAGAAAGATGCGATGGTGCCGATGGGCGGCCTGCTGTGC  
ATGAAAGACGACAGCTTCTTTGATGTGTACACCGAGTGCAGAACCCTTTGCGTGGTGCAGGAAGGCTTCCCCGACA  
TATGGCGGCCTAGAAGGCGGCGCGATGGAGCGTCTGGCGGTAGGTCTGTATGACGGCATGAATCTCGACTGGCTG  
GCTTATCGTATCGCGCAGGTACAGTATCTGGTCGATGGTCTGGAAGAGATTGGCGTTGTC

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## Appendice 2 : sequence used to inactivate the tryptophanase *tnaA* gene by replacement with the *trpR* gene (construction of ICONE 200)

Nucleotides 1 to 510 : sequence of the *E. coli tnaA* promoter

Nucleotides 511 to 837 : sequence of the *E. coli trpR* gene

Nucleotides 511 to 513 and 835 to 837 respectively : start and stop codons of the *E. coli trpR* gene

Nucleotides 838 to 843 : PstI restriction site inserted downstream to the stop codon of *trpR*

Nucleotides 844 to 1468 : sequence of the *E. coli* genome downstream to the *tnaA* coding sequence

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CTGTCAGATGCGCTTCGCTTCATTGTTACCGCTCCTGTTATTCCTCAACCCTTTTTTTAAACATTAAAATTCTTA
CGTAATTTATAATCTTTAAAAAAGCATTTAATATTGCTCCCCGAACGATTGTGATTTCGATTCACATTTAAACAA
TTTCAGAATAGACAAAAACTCTGAGTGTAATAATGTAGCCTCGTGTCTTGCGAGGATAAGTGCATTATGAATATC
TTACATATATGTGTGACCTCAAATGGTTCAATATTGACAACAAAATTGTCGATCACCGCCCTTGATTTGCCCTT
CTGTAGCCATCACCGAGCCAAACCGATTGATTCAATGTGTTCTATTTGTTTGCTATATCTTAATTTGCCCTTT
GCAAAGGTCATCTCTCGTTTATTTACTTGTTTTAGTAAATGATGGTGCTTGCATATATATCTGGCGAATTAATCG
GTATAGCAGATGTAATATTACAGGGATCACTGTAATTAATAAATGAAGGATTATGTAATGGCCCAACAATCA
CCCTATTTCAGCAGCGATGGCAGAACAGCGTCACCAGGAGTGGTTACGTTTTGTCGACCTGCTTAAGAATGCCTAC
CAAAACGATCTCCATTTACCGTTGTAAACCTGATGCTGACGCCAGATGAGCGCGAAGCGTTGGGGACTCGCGTG
CGTATTGTGCAAGAGCTGTTGCGCGGCGAAATGAGCCAGCGTGAGTTAAAAAATGAACTCGGCGCAGGCATCGCG
ACGATTACGCGTGGATCTAACAGCCTGAAAGCCGCGCCCGTCGAGCTGCGCCAGTGGCTGGAAGAGGTGTTGCTG
AAAAGCGATTGACTGCGAGTTAATACTACAGAGTGGCTATAAGGATGTTAGCCACTCTCTTACCCTACATCCTCAA
TAACAAAAATAGCCTTCCTCTAAAGGTGGCATCATGACTGATCAAGCTGAAAAAAGCACTCTGCATTTTGGGGT
GTTATGGTTATAGCAGGTACAGTAATTGGTGGAGGTATGTTTGCTTTACCTGTTGATCTTGCCGGTGCTGGTTT
TTCTGGGGTGCCCTTATCCTTATCATTGCCTGGTTTTCAATGCTTCATTCCGGGTATTGTTATTAGAAGCAAAT
TTAAATTATCCCGTCGGCTCCAGTTTTAACACCATCACCAAAGATTTAATCGGTAACACCTGGAACATTATCAGC
GGTATTACCGTTGCCTTCGTTCTCTATATCCTCACTTATGCCTATATCTCTGCTAATGGTGCGATCATTAGTGAA
ACGATATCAATGAATTTGGGTATCACGCTAATCCACGTATTGTGCGGGATCTGCACAGCCATTTTCGTTGCCAGC
GTATTGTGGTTAAGTTCGTTAGCCGCCAGTCGTATTACCTCATTGTTCCCTCGGGCTGAAGATTATCTCCTTTGTG
ATCGTGTTTGGTTCTTTTTCTTCCAGGTCGATTACTCCATTC
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